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Expression of glucocorticoid receptor in the intestine of a euryhaline teleost, the Mozambique tilapia (*Oreochromis mossambicus*): Effect of seawater exposure and cortisol treatment

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Abstract

Cortisol plays an important role in controlling intestinal water and ion transport in teleosts possibly through glucocorticoid receptor (GR) and/or mineralocorticoid receptor. To better understand the role of GR in the teleost intestine, in a euryhaline tilapia, *Oreochromis mossambicus*, we examined (1) the intestinal localizations of GR; (2) the effects of environmental salinity challenge and cortisol treatment on GR mRNA expression. The mRNA abundance of GR in the posterior intestinal region of tilapia was found to be higher than that in the anterior and middle intestine. In the posterior intestine, GR appears to be localized in the mucosal layer. GR mRNA levels in the posterior intestine were elevated after exposure of freshwater fish to seawater for 7 days following an increase in plasma cortisol. Similarly, cortisol implantation in freshwater tilapia for 7 days elevated the intestinal GR mRNA. These results indicate that seawater acclimation is accompanied by upregulation of GR mRNA abundance in intestinal tissue, possibly as a consequence of the elevation of cortisol levels. In contrast, a single intraperitoneal injection of cortisol into freshwater tilapia decreased intestinal GR mRNA. This downregulation of the GR mRNA by cortisol suggests a dual mode of autoregulation of GR expression by cortisol.

Keywords: Cortisol; Receptor; Intestine; Osmoregulation; Seawater adaptation

Introduction

Cortisol, a major glucocorticoid, possesses important osmoregulatory, metabolic and immune functions in teleost fishes (Wendelaar Bonga, 1997; Mommsen et al., 1999). Perhaps, the most intensively studied aspect of cortisol's actions in teleosts is its osmoregulatory functions in euryhaline species. At the intestine, cortisol increases water and ion absorption in seawater teleosts, which is necessary to offset the dehydrating effects of the seawater environment. This is achieved by an increase in ion and water permeability as well as by an increase in the active uptake of ions, especially chloride transport (Karnaky, 1998; Hirano, 1991), which increases the osmotic uptake of water across the intestinal mucosa (Loretz, 1995). As has been reported for gills (McCormick, 1995, 2001; Sakamoto et al., 2001), cortisol also increases levels of Na^+,K^+-ATPase activity in the intestine (see Wendelaar Bonga, 1997 for review).

The physiological response to cortisol is mediated through binding to its receptor(s). To clarify the role of the receptors, radioligand saturation binding and expression of glucocorticoid receptor (GR) have been studied mainly in the gill, elements of the immune system and the liver. Surprisingly, less attention

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has been paid to the intestine, although regulation and function of cortisol receptors as well as the subsequent tissue/cell response to cortisol have been variable (Pottinger, 1990; Pottinger et al., 1994, 2000; Maule and Schreck, 1991; Shrimpton and Randall, 1994; Uchida et al., 1998; Mommsen et al., 1999; Shrimpton and McCormick, 1999; Dean et al., 2003; Greenwood et al., 2003; Sathiyaa and Vijayan, 2003; Vijayan et al., 2003). Furthermore, involvement of GR in teleost osmoregulation has now become controversial, since mineralocorticoid receptors, which are important in the control of hydro-mineral balance in higher vertebrates (Johnson, 1992), have been recently identified in teleost fish to be more sensitive to cortisol than GR (Colombe et al., 2000; Greenwood et al., 2003; Sturm et al., 2004).

Given the unclear role of GR in teleost osmoregulation, together with the lack of information on the cortisol receptor in the intestine, an important osmoregulatory organ, the objective of the present investigation was to evaluate GR expressions in the intestine of euryhaline Mozambique tilapia. Our approach was (1) to examine the localization of GR in the intestine; (2) to measure GR receptor mRNA in the intestine after transfer of freshwater tilapia to seawater; and (3) to examine the effects of chronic and acute cortisol treatment on GR mRNA expression in the intestine.

Materials and methods

Animals

Oreochromis mossambicus comprising both sexes were reared in outdoor tanks at a density of 10 fish (30–40 g) per tank (100 l), supplied with a continuous flow of freshwater under a natural photoperiod at the Hawaii Institute of Marine Biology, University of Hawaii, Manoa as described by Kajimura et al. (2003, 2004). Water temperature was maintained at 25±2 °C. Animals were fed daily with ProForm (Agro Pacific, Chilliwaeck, BC, Canada), approximately 2% of body weight per day.

The sampling consisted of rapidly netting the fish and anaesthetizing the fish with a lethal dose of MS-222 (100 mg/l). The fish were weighed and the intestinal segments were sampled following the methods described by Mainoya (1982). Briefly, the portions of intestine that loop around the liver were sampled (anterior segments). The remaining portion (excluding the terminal of the intestine) was also removed and divided into two segments of equal length (middle and posterior segments).

Northern blot analyses

The removed intestinal segments were snap-frozen in liquid nitrogen and stored at −80 °C for analysis. Total RNA was extracted from the individual segments according to the method of Chomczynski and Sacchi (1987) using an RNA purification kit (Isogen; Wako Chemical, Osaka, Japan). The RNA was quantified by spectrophotometry. Electrophoresis was performed, by loading 10 μg total RNA per lane in a 1% agarose-formaldehyde gel, followed by transfer to a nylon membrane (Amersham Biosciences, Arlington Heights, Illinois, USA) by capillary blotting (Sambrook et al., 1989). The RNA was covalently attached to the membrane by baking at 80 °C for 2 h and by UV cross-linking. cDNAs for tilapia GR hormone-binding domain (Tagawa et al., 1997: accession no. D66874) and the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH, NM002046) were labeled with [^{32}P]dCTP (BcaBEST DNA labeling kit, Takara). The membranes were hybridized with the probe for GR or GAPDH for 18 h following prehybridization, in Perfecthyb hybridization solution according to the manufacturer's instructions (Stratagene). Following hybridization, membranes were washed in 2 × SSC and 0.1% SDS at room temperature for 2 × 10 min. They were washed again with 1 × SSC containing 0.1% SDS at 65 °C for 2 × 20 min, 0.1 × SSC containing 0.1% SDS for 20 min at 65 °C, and then rinsed at room temperature. The membranes were exposed to phosphor imaging plate (Fuji imaging plate-Bas III, Tokyo, Japan). Intensity of the hybridization signals was assessed with an Auto Image Analyzer (Bas 2000, Fuji Film, Tokyo, Japan). After analysis of messages corresponding to GR, the membranes were dehybridized as described by the manufacturer. To confirm complete removal of the GR probe, membranes were re-exposed to phosphor screens to ascertain if any remaining signal was present. The membranes were then rehybridized to the GAPDH probe. Serial dilution of the RNA demonstrated linearity between the amount of RNA and hybridization signals obtained (data not shown). Messenger RNA data are represented in arbitrary units normalized to the relatively constant quantity of GAPDH signal normalized to the quantity of GAPDH signals, and pooled RNA was used as an internal standard to adjust the variability among the blots. Molecular sizes were estimated relative to migration of an RNA size marker (Toyobo, Osaka, Japan). GR mRNA abundance was predominant in posterior half of the intestinal segments remaining after removal of the anterior segments, and these regions were analyzed thereafter.

GR location in the intestine

Immunocytochemical staining was carried out by the avidin–biotin peroxidase complex (ABC) method (Hsu et al., 1981) using Vectastain ABC Kit (Vector Laboratories, Burlingame, CA) basically as described in Uchida et al. (1996) and Sakamoto et al. (2005). The intestine was fixed overnight in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4 °C. The posterior part of the intestine was dehydrated in ethanol and embedded in paraplast. Sections were cut at 5 μm on a microtome and attached to 3-aminopropyltriethoxysilane-coated slides. The sections were immunocytochemically stained with a polyclonal antibody raised against a synthetic peptide corresponding to hydrophilic portion of the hormone binding domain of tilapia GR (Tagawa et al., 1997). This primary antiserum diluted 1:1800 in a solution containing 0.5% Triton X-100 and 1% bovine serum albumin (BSA, Sigma, Tokyo) was applied to sections overnight at 4 °C. To test the specificity of the immunoreaction, controls omitting the GR primary antiserum were performed. Furthermore, the immu-

noreaction was decreased in the cortisol-injected fish, and cellular localization of the immunoreaction was in accordance with cellular expression of the GR gene identified by in situ hybridization (Uchida et al., 1997).

Salinity transfer

The salinity of some tanks was changed from freshwater to full-strength seawater (32.5 ppt) without disturbing the fish, by closing the freshwater valve and the opening seawater valve. It took about 30 min for complete replacement. Other fish that were retained in freshwater tanks served as controls.

Chronic cortisol treatment

The freshwater fish were mildly anaesthetized with MS-222 (50 mg/l, buffered with $NaHCO_3$). We made a small incision in the dorsal skin, gave the subcutaneous implant of either a cholesterol pellet alone (control) or cholesterol pellet containing 2.5 or 25 μg cortisol (Na-hydrocortisone-21-hemisuccinate) /g body mass into the muscle, and sutured the wound to seal it as described by Takagi et al. (1992) and Yada et al. (1999). As a reference, some fish were also treated with estradiol. With intact freshwater fish, these fish were sampled 7 days later, when each fish was examined and the presence (weight) or absence of implanted pellets recorded.

Acute cortisol treatment

The freshwater fish were mildly anaesthetized with MS-222 (50 mg/l, buffered with $NaHCO_3$) and injected intraperitoneally with cortisol at doses of 1 or 10 μg/g in a volume of 5 μl/g. Control fish received vehicle (0.9% NaCl, 0.5% bovine serum albumin) only. Samples were taken at 1 day post injection.

Determination of plasma variables

The sampling consisted of rapidly netting at least four fish from each tank and anaesthetizing the fish with a lethal dose of MS-222 (100 mg/l) between 14:00 h and 16;00 h. Before the sampling of the intestines, blood was collected from the caudal vessels by a needle and syringe treated with ammonium heparin (200 U/ml; Sigma, St. Louis, MO). Immediately after blood sampling, plasma was separated by centrifugation (10,000×*g* for 5 min) and stored at −80 °C until analyzed.

Cortisol concentrations in plasma were measured by a validated enzyme immunoassay (Sakamoto et al., 2002). In brief, plasma was extracted with ether. In plate wells (coated with mouse-anti-rabbit IgG), standards and samples (50 μl) were incubated with 50 μl cortisol-21-succinate linked to horseradish peroxidase and 50 μl antiserum to cortisol for 1.5 h at room temperature. After washing, 150 μl of substrate solution was added, incubated for 1 h at 37 °C and absorbance was measured at 492 nm on a plate reader (MTP-120, Corona Electric, Ibaragi, Japan). The intra-assay coefficient of variation was 2%, and the inter-assay coefficients of variation were 10% for cortisol. Plasma osmolality was measured using a Wescor 5500 vapor pressure osmometer (Wescor, Inc., Logan, Utah, USA). Plasma Na^+ was determined by atomic absorption spectrophotometry (Z5300; Hitachi, Tokyo, Japan).

Statistics

Statistical significance of differences among mean values was tested using an analysis of variance (ANOVA) followed by the least significant difference test with Statview 4.11 (Abacus Concepts, Japan).

Results

GR localization in the intestine

Fig. 1 shows GR transcripts at 6.5 kb from the intestine of tilapia. GR mRNA abundance was predominant in posterior half of the intestinal segments remaining after removal of the anterior segments, and these regions were analyzed thereafter. The signals are not due to blood cell contamination, as little amount of RNA was extracted from blood.

Fig. 2a shows specific immunoreactive GR in the posterior intestine. Immunoreactive GR was observed in the epithelial cells. Less immunoreactivity was found in the controls performed to validate the specificity of the immunoreaction (Fig. 2b).

Salinity transfer

No mortality was seen after transfer of the fish. Plasma osmolality peaked at 590±11 mOsm/kg in seawater fish 1 day after transfer, a value significantly higher than the 325±2.2 mOsm/kg value measured in freshwater fish ($p<0.01$).

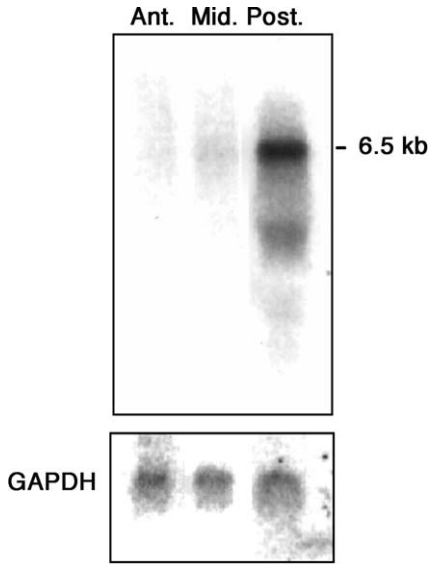

Fig. 1. Representative results of Northern blots performed with 10 μg of total RNA from the freshwater-adapted tilapia anterior, middle and posterior intestine. Blots were hybridized with cDNA probe of tilapia GR, and rehybridized with GAPDH (glyceraldehyde-3-phosphate dehydrogenase) cDNA as described.

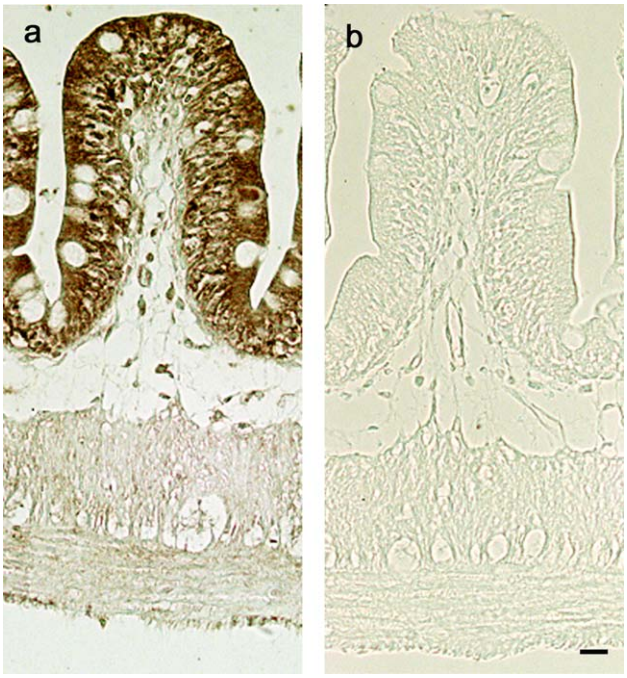

Fig. 2. Cellular localization of GR in the tilapia posterior intestine. GR immunostaining was present specifically in the cytoplasma of epithelial cells (a). The negative control equivalent of part (a) in which the GR antiserum was omitted (b). Scale bar=10 μm.

Seawater fish osmolality dropped by 7 days after seawater exposure, yet remained significantly ($p<0.05$) higher than levels seen in freshwater controls. Plasma sodium levels showed a profile that was similar to that for plasma osmolality. The change in plasma cortisol was also similar: plasma cortisol was increased after seawater transfer; these values were higher than that in freshwater (Fig. 3a). There was a significant ($p<0.01$) elevation in GR mRNA abundance in the intestine after seawater transfer (Fig. 3b).

Chronic cortisol treatment

Plasma cortisol levels were elevated in the fish implanted with cortisol significantly ($p<0.05$) at a dose of 25 μg/g but not in fish implanted with 5 μg/g cortisol relative to the sham group (Fig. 4a). Mean levels of intestinal GR mRNA abundance were higher in fish implanted with cortisol, and those animals implanted with 25 μg/g exhibited significantly ($p<0.01$) greater GR mRNA levels than the sham group (Fig. 4b). Estradiol treated with the same protocols/doses was without a significant effect (data not shown). There were no significant differences between the sham group and intact controls.

Acute cortisol treatment

Plasma cortisol was increased by injections of cortisol at doses of 1 μg/g and 10 μg/g ($p<0.01$) relative to vehicle-injected controls (Fig. 5a). Mean levels of intestinal GR mRNA abundance were lower in both groups of cortisol-injected fish, compared with the controls, and this cortisol-dependent decrease was significant in those fish injected with the high dose (10 μg/g of cortisol, Fig. 5b). There were no significant differences between the vehicle-injected and intact controls.

Discussion

Herein, we report a significant rise in GR mRNA abundance in the intestine of a euryhaline teleost during the process of seawater adaptation. Based on cortisol's well-documented roles in differentiating the intestinal functions to seawater (Hirano, 1991; Wendelaar Bonga, 1997), this induction of GR expression would appear to be an important component in cortisol's hypoosmoregulatory functions. Furthermore, the epithelia of the posterior intestine, where GR expression was most prominent (Figs. 1 and 2), have been suggested to be more important in water absorption than those of the other regions of intestine in tilapia (Mainoya, 1982), although the transport capacity was not examined in the present study. In the different regions of the tilapia intestine where GR transcript was almost undetectable, metabolic effects were also reported after treatment with an intraperitoneal cortisol deposit (Mommsen et al., 2003a,b). In these regions, cortisol may act indirectly through other metabolic hormones.

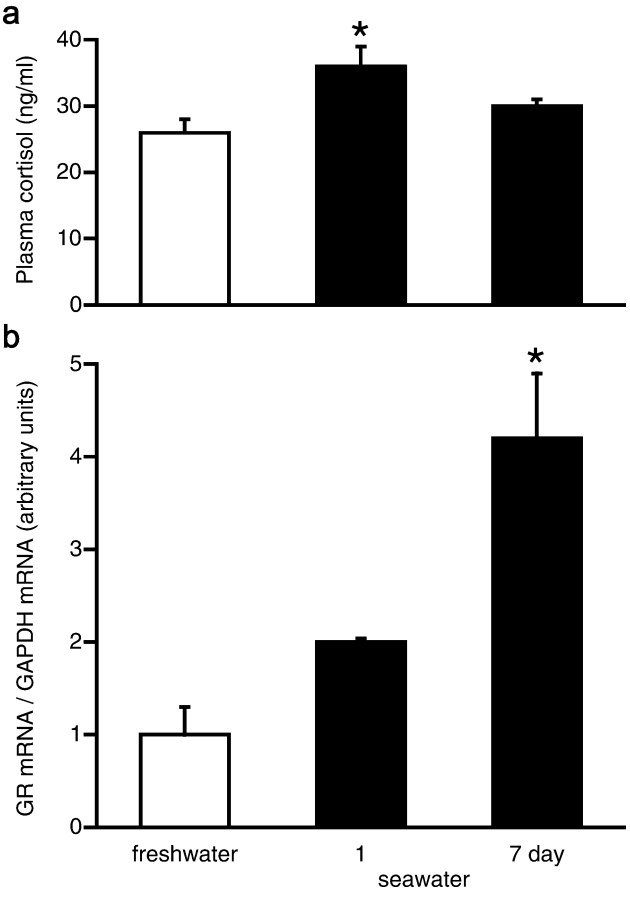

Fig. 3. Plasma cortisol levels (a) and glucocorticoid receptor (GR) mRNA abundance in the posterior intestine (b) of freshwater tilapia transferred to either freshwater or seawater for 7 days. Messenger RNA levels were expressed as the ratio to GAPDH mRNA (arbitrary units). Values are expressed as means±SEM ($n=4–8$). Significant differences (ANOVA followed by the least significant difference test; $p<0.05$) from freshwater fish are indicated by asterisks.

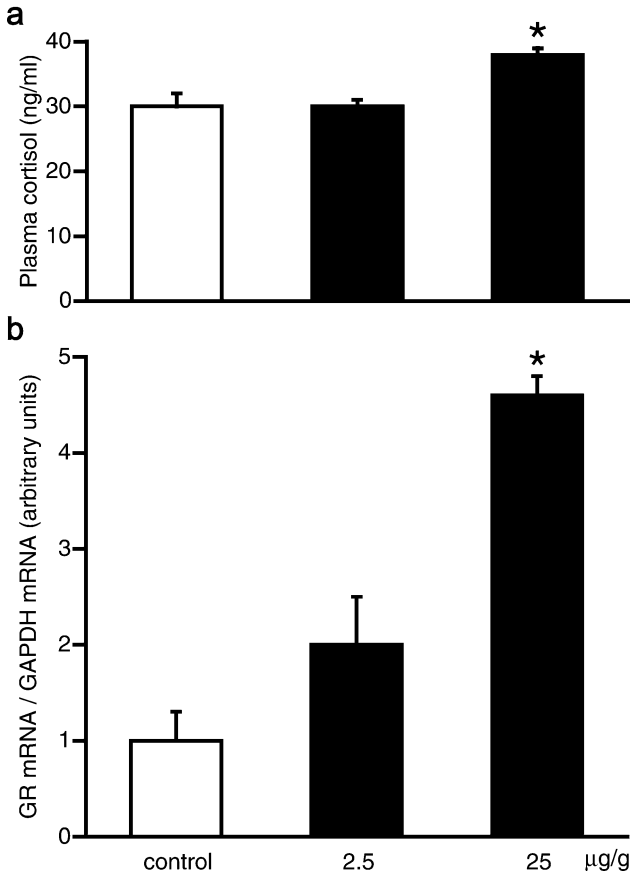

Fig. 4. Effect of implantation of cortisol for 7 days on plasma cortisol levels (a) and GR mRNA abundance in the intestine (b) of freshwater tilapia. Messenger RNA levels were expressed as the ratio to GAPDH mRNA (arbitrary units). Values are expressed as means ± SEM ($n = 4–5$). Significant differences (ANOVA followed by the least significant difference test; $p < 0.05$) are indicated by asterisks.

Although we have observed increases in plasma cortisol during seawater adaptation in agreement with the well-documented role as a seawater adapting hormone in tilapia and other fish (McCormick, 2001), other reports on blood cortisol in seawater-challenged tilapia have been inconsistent (Morgan et al., 1997; Dean et al., 2003; Kajimura et al., 2004). Despite the unaltered plasma level, Balm et al. (1995) reported that the total volume of *O. mossambicus* interrenal cells increased 10-fold in seawater-adapted fish and was paralleled by an increase in in vitro interrenal cortisol production, indicating a simultaneous elevation in the production/secretion and clearance of cortisol in seawater-acclimated tilapia. Therefore, our long-term cortisol implantation study seems to mimic the cortisol secretion levels seen during seawater challenge. Indeed, plasma cortisol levels in cortisol-implanted fish, not in cortisol-injected fish, were within the range of values seen in the seawater-transferred tilapia. Thus, the increase in intestinal GR mRNA abundance during seawater adaptation may be linked to the chronic elevation in cortisol secretion, since cortisol implantation also induced the intestinal GR (Fig. 3b).

Interesting differences in the regulation of GR mRNA have emerged from the two cortisol manipulations conducted in this study. Especially, we observed increases in intestinal GR mRNA in the chronic treatment, which are similar to recent reports using the rainbow trout liver (Sathiyaa and Vijayan, 2003; Vijayan et al., 2003). On the other hand, the acute injection of cortisol at the high dose, by contrast with the changes observed after salinity transfer or cortisol implantation, resulted in a significant decrease in GR mRNA levels, which corresponds with findings in mammalian studies wherein reduced GR transcription was reported after cortisol treatment (Rosewicz et al., 1988; Yudt and Cidlowski, 2002). The upregulation of GR following salinity challenge and cortisol implantation may be a feature that allows cortisol to elicit its chronic actions, such as regulation of tissue differentiation, development and metabolism. The downregulation of the GR mRNA, seen in the cortisol injection experiment, may represent a mechanism to decrease tissue responsiveness to acute cortisol stimulation, which may be analogous to situations following acute stress. Experiment to stress fish by physical disturbance and to exposure of seawater-acclimated fish to cortisol may clarify these differences in the GR regulation. As a potential regulatory mechanism, the different effects of cortisol via multiple receptors (GR, mineralocorticoid receptor and membrane bound receptors) as well as the model of allostasis may be addressed (McEwen and Wingfield, 2003).

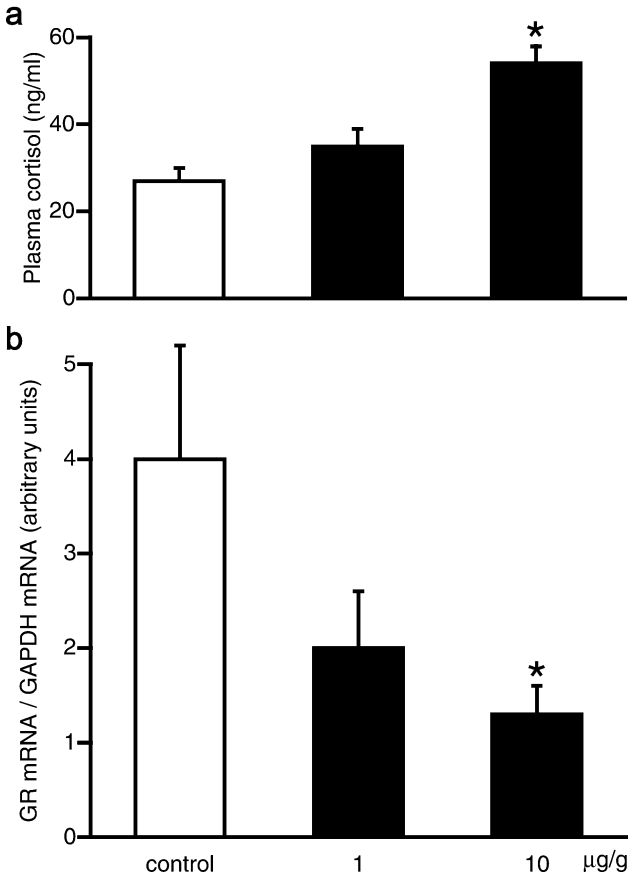

Fig. 5. Effect of single cortisol injection on plasma cortisol levels (a) and GR mRNA abundance in the intestine (b) of freshwater tilapia. Messenger RNA levels are expressed as the ratio to GAPDH mRNA (arbitrary units). Values are expressed as means ± SEM ($n = 4–5$). Significant differences (ANOVA followed by the least significant difference test; $p < 0.05$) are indicated by asterisks.

Herein, we have shown that GR expressions in the intestinal epithelia of euryhaline tilapia are altered by seawater adaptation and cortisol manipulations. While these changes are consistent with the known hypoosmoregulatory actions of cortisol in euryhaline teleosts, this is the first study to document such changes in this important osmoregulatory organ. Nevertheless, further investigations are necessary to determine whether the changes in GR mRNA abundance actually reflect changes in the protein content. Most mammalian studies showed a positive correlation between GR mRNA abundance and protein content, but a mismatch in GR gene and protein responses has been recently observed in rainbow trout hepatocytes (Sathiyaa and Vijayan, 2003; Vijayan et al., 2003). Furthermore, in addition to the presence of multiple GR genes and their mRNA splice variants, a mineralocorticoid receptor has been recently identified to bind cortisol (Takeo et al., 1996; Colombe et al., 2000; Marsigliante et al., 2000; Bury et al., 2003; Greenwood et al., 2003; Sturm et al., 2004). In ion homeostasis at the intestine, mineralocorticoid receptor may be important, whereas GR should play a distinct role during seawater adaptation. For example, GR may be involved in the apoptosis, as reported for carp leukocytes (Weyts et al., 1998). We have recently found that induction of epithelial cell apoptosis in the intestine appears to play an important role in increasing the intestinal epithelial permeability during adaptation of euryhaline fish to seawater, and that treatment of cortisol, not 11-deoxycorticosterone (the putative teleostean mineralocorticoid, Sturm et al., 2004) stimulated the intestinal apoptosis (our unpublished observations).

Conclusion

Seawater acclimation of tilapia is accompanied by upregulation of GR mRNA abundance in the epithelia of the posterior intestine, possibly as a consequence of the elevated cortisol levels, suggesting the importance of cortisol-intestinal GR in seawater adaptation. In contrast, a single intraperitoneal injection of cortisol into tilapia decreased the intestinal GR mRNA, which proposes a dual autoregulation of GR expression by cortisol.

Acknowledgments

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